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Immune complex binding efficiency of erythrocyte complement receptor 1 (CR1)

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SUMMARY

C3b-coated immune complexes adhere to the complement receptor 1 (CR1, CD35) on human erythrocytes. This multi-valent binding might be favoured by the known clustering of CR1 and by the multiple C3b-binding sites on each CR1. The size of the CR1 clusters correlates directly with the number of CR1/erythrocytes, and the different structural CR1 alleles bear between two and five C3b-binding sites. Using radiolabelled hepatitis B surface antigen–antibody complexes, we investigated whether CR1 numbers and structural alleles modulate the ability of erythrocytes to bind immune complexes, and assessed if any reorganization of immune complexes takes place at the erythrocyte surface after the initial binding reaction. The binding efficiency (immune complexes/CR1) correlated with CR1 number as determined by the maximal binding at 4°C, the kinetics of binding at 37°C, and the binding in the presence of excess immune complexes and of immune complexes of small size. Binding efficiencies were similar for erythrocytes with low CR1 from normal subjects and patients with AIDS or SLE. A monoclonal antibody blocking the C3b-binding sites (3D9) of CR1 interfered with binding efficiency at a lower concentration on cells bearing low CR1 numbers, suggesting that CR1 clustering is essential. The larger alleles of CR1 (DD and BB) were more efficient than AA alleles. The distribution of immune complexes, visualized by immunofluorescence, was heterogeneous on erythrocytes: about two out of three cells bore between one and 12 immune complexes. No visible immune complex reorganization took place after initial binding, as prefixed erythrocytes displayed the same immune complex distribution and number/erythrocytes as unfixed erythrocytes. The contribution of CR1 alleles in immune complex binding efficiency was confirmed by morphological analysis. These results demonstrate that immune adherence efficiency is the resultant of the CR1 clustering, as well as the particular alleles carried by erythrocytes. Moreover, there is little or no immune complexes surface reorganization after the initial binding reaction.

Keywords complement receptor 1 (CD35) erythrocytes immune complexes immune adherence

INTRODUCTION

CR1 at the surface of erythrocytes are responsible for the C3b-mediated immune adherence reaction, first described by Nelson (1953) for bacteria. It is known that the mean number of CR1 per erythrocytes varies widely within the normal population (100–1000 CR1/erythrocytes), and is genetically determined (Wilson *et al.*, 1982). Erythrocyte CR1 are also diminished in various autoimmune diseases and AIDS (Ross *et al.*, 1985; Tausk *et al.*, 1986). Such a low CR1 number might affect the transport and inactivation of circulating immune complexes, which under normal circumstances adhere to erythrocytes before being cleared by the fixed macrophages of liver and spleen (Cornacoff *et al.*, 1983; Waxman *et al.*, 1986; Schifferli *et al.*, 1988).

In vitro, when measured at equilibrium (90 min of incubation at 37°C), the binding of pre-opsonized immune complexes to erythrocytes correlates directly with their CR1 number (Ng, Schifferli & Walport, 1988). However, different results have been obtained *in vivo* in humans (Schifferli *et al.*, 1989). A fraction of tetanus toxoid (TT)/antibody complexes injected intravenously bound very rapidly to erythrocytes. Under the experimental conditions used, CR1 were in vast excess in the circulation to bind the small quantities of injected immune complexes. Thus, it was surprising to find that the adherence of immune complexes was directly proportional to the CR1 number per erythrocyte. Obviously, equilibrium is never reached *in vivo*: the binding of immune complexes to CR1 is immediately followed by their release due to inactivation of C3b

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by factor I (Medof *et al.*, 1982). Thus, the observed rate and magnitude of immune complex adherence is the resultant of ongoing binding and release reactions. However, factor I concentrations were not responsible for the variations observed, indicating that the limiting factor was to be found in the binding reaction.

Adherence of immune complex to erythrocytes requires a multi-valent interaction between C3b and CR1, since the affinity of monomeric C3b for CR1 is too low to account for it (Arnaout *et al.*, 1981). On these cells, a fraction of the CR1 form small clusters of different sizes (from three to 15 CR1/cluster) (Paccaud, Carpentier & Schifferli, 1988). This clustering is more prominent on erythrocytes with high CR1 numbers. Another important aspect is that each CR1 has multiple binding sites for C3b (Bartow, Klickstein & Fearon, 1989), which might further improve the multi-valent binding reaction. Indeed, the extracellular domaine of CR1 is made of a series of long homologous repeats (LHR), and except for the LHR proximal to the cell membrane, each LHR bears a C3b binding site. Four structural alleles of CR1 have been defined: C, A, B and D (two to five binding sites, respectively). These alleles differ in the number of LHR (Wong *et al.*, 1989), i.e. in the number of C3b binding sites. Thus, CR1 number and alleles might both determine the efficiency of immune adherence.

Here, the adherence of hepatitis B surface antigen–antibody complexes to erythrocytes was examined *in vitro* to define the role of CR1 number and/or alleles in immune complex binding. In addition, we assessed whether any reorganization of immune complexes takes place at the erythrocyte surface after the initial binding reaction.

MATERIALS AND METHODS

Immune complexes

Hepatitis B surface antigen (HBsAg) was kindly provided by Dr D. Labert (Pasteur Vaccins, Val de Reuil, France). The HBsAg (molecular weight of 3000 kD) was dialysed against phosphate-buffered saline (PBS), pH 7·4, and radiolabelled with the Bolton and Hunter reagent (*N*-succinimidyl-3-(4-hydroxy,5-[^{125}I] iodophenyl) propionate) (Amersham International, Amersham, UK) to a specific activity of 2·3 μCi ^{125}I/μg. The antibody was polyclonal IgG anti-HBsAg obtained from blood donors, at a concentration of 50 U/ml (Immunoglobulin anti-hepatitis B SRK; Swiss Red Cross, Bern, Switzerland). Complexes were formed *in vitro* in large antibody excess, by mixing 50 μg of ^{125}I-HBsAg with 1140 U of anti-HBsAg, and incubating the mixture at 37°C for 90 min. The soluble immune complexes formed (no precipitation after centrifugation for 10 min at 4000 ***g*** were mixed with 500 μl normal human serum and incubated for 15 min at 37°C. The complement-opsonized immune complexes were then partially purified by sucrose density gradient ultracentrifugation (Schifferli *et al.*, 1988). The first five fractions containing the largest immune complexes were pooled and dialysed against PBS before use. For some experiments, smaller immune complexes were selected (fractions 6–10). These preparations were essentially free of factor I (<1% factor I by immunodiffusion); there was less than 5% release of immune complexes from erythrocytes between 2 h and 24 h of incubation at 37°C. Control immune complexes were prepared similarly using heat-inactivated human serum (63°C for 2 h).

Erythrocytes and immune adherence (IA) assays

Erythrocytes from fresh EDTA-anti-coagulated blood were washed five times in cold PBS, removing the buffy coat between each wash. Complement-opsonized and control immune complexes were incubated with erythrocytes from different donors at various temperatures, concentrations and periods of time. The binding of immune complexes to erythrocytes was determined by counting erythrocyte pellets and supernatants after separation through oil (Ng *et al.*, 1988), and by immunofluorescence (IF) after washing the cells with cold PBS three times.

When needed, washed erythrocytes were fixed in 1% (w/v) paraformaldehyde in PBS for 10 min at 4°C, or immediately fixed *ex vivo* by adding 500 μl of freshly drawn blood into 50 ml of ice-cold 1% paraformaldehyde/PBS. Similar procedures were performed to fix cells with 1% ethyldimethylaminopropyl carbodimide/0·2% glutaraldehyde for 7 min followed by cold PBS containing 0·5 mg/ml $NaBH_4$ for 15 min.

CR1

Erythrocyte CR1 numbers were measured as previously described (Ross *et al.*, 1985) using a mouse monoclonal antibody (MoAb) IgG1 anti-CR1, E11 (kindly provided by Dr N. Hogg) radiolabelled to a specific activity of 0·47 μCi/μg using the iodogen method (Fraker & Speck, 1978). CR1 sites were blocked with 3D9, a MoAb known to inhibit C3b binding, kindly provided by Dr J. Atkinson.

CR1 structural alleles were defined following the method of Mitchell & Sim (1989) with some modifications. In brief, packed erythrocytes were lysed in 0·05 M Tris–HCl, pH 6·8, containing 5 mM DFP, 5 mM PMSF and 10 mM EDTA for 20 min on melting ice. After three washes in the same buffer, the erythrocyte membranes were dissolved in 0·07 M Tris–HCl, pH 6·8, 4% SDS, 10% glycerol, 0·002% bromophenol blue and 0·5% CHAPS for 30 min at room temperature. The samples were run on a 5–15% SDS–PAGE gel under non-reducing conditions, and blotted on to nitrocellulose. After blocking the nitrocellulose with 3% low-fat powdered milk, it was incubated overnight at 4°C with 50 μg of E11 or 3D9. The MoAbs were revealed with further incubations with rabbit immunoglobulin anti-mouse immunoglobulin (Dako, Glostrup, Denmark) followed by ^{125}I-protein-G (Amersham). Out of 60 determinations, 34 were AA, and some of those were used for comparing erythrocytes with different CR1 number. One BB and one DD allele was used for comparing the effects of alleles, using AA controls with similar CR1 numbers. Most were normal individuals; however, five SLE subjects and three AIDS subjects with low CR1 were included to examine the effects of acquired low CR1 number.

Microscopy

Erythrocytes were incubated with immune complexes in a final volume of 100 μl, washed in PBS, before adding rabbit IgG anti-human C3 for 30 min at room temperature (Paccaud *et al.*, 1988). This was followed by a wash in PBS, a 30-min incubation with biotinylated protein-A (Amersham), one wash in PBS, an incubation 30 with the streptavidin–FITC or streptavidin–Texas red (Amersham), and three final washes in PBS. The cells were applied to poly-L-lysine-coated slides and allowed to settle. Unbound cells were washed and the slides were mounted in PBS/50% glycerol to which 25 mg/ml diazabicyclo-octan were added to prevent bleaching. Radiolabelled immune complexes were used to assess possible loss of immune complexes after their

initial binding by the different incubations: the drop in radioactivity was less than 5%. Controls included the use of non-opsonized immune complexes, or omission of immune complexes, rabbit anti-human C3, biotinylated protein A and streptavidin–FITC or streptavidin–Texas red. They validated the specificity of the method. Preparations were photographed at a final magnification of ×350 on an Olympus BH-2 microscope equipped with epifluorescence (exitation filters IF490 and EY455). Erythrocytes were first visualized by phase contrast, and the number of fluorescent dots determined on at least 120 cells. However, the fluorescent intensity of each dot was not taken into account: this might introduce systematic errors when more than one immune complex are localized near each other. Yet, as the size and content in C3 fragments might vary from one immune complex to another, it was considered not appropriate to record this variable.

In some experiments, cells were processed for electron microscopy. After incubation with immune complexes and rabbit anti-human C3, the cells were incubated for 30 min with 10 nm gold-labelled goat anti-rabbit IgG, and washed three times in PBS. The cells were sedimented on a poly-L-lysine-treated plastic coverslip for 5 min and non-adherent cells washed with PBS. The erythrocytes were post-fixed with 2% glutaraldehyde/PBS for 10 min, dehydrated in ethanol, and processed for label-fracture exactly as described (Pinto da Silva & Kan, 1984). For each condition, at least 50 erythrocytes were examined.

All experiments were done in duplicate.

RESULTS

Since our goal was to compare the efficiency of immune complexes binding to CR1 in different populations of erythrocytes, most experiments were performed at equal CR1 concentration. First, the maximum binding of immune complexes was determined after incubation for 2 h at 37°C in the presence of excess CR1 (Fig. 1b). This binding was very similar for the four types of erythrocytes tested. However, the results differ broadly when the same assay was performed at 4°C: a clear correlation between binding and CR1 number became apparent (Fig. 1a). When the same samples were incubated for 24 h and 48 h, intermediate results were obtained (not shown), but binding at 4°C never reached the values obtained at 37°C. These experiments were all performed with immune complexes of very large size. Using immune complexes of smaller size, it was possible to demonstrate differences in immune adherence even at 37°C (Fig. 1d). Interestingly, erythrocytes from patients with AIDS or SLE, having low CR1 numbers, were capable of binding immune complexes with similar efficiency to normal cells with low CR1.

The next step was to study the kinetics of the binding reaction of the large immune complexes at 37°C (Fig. 2): the binding reaction was slower for erythrocytes with low CR1 numbers from a healthy individual and a patient with AIDS, than for erythrocytes with high CR1 number.

To assess the binding of immune complexes to CR1 at increasing CR1 concentration, an intermediate time-point was selected. Under such non-equilibrium conditions, immune complexes bound more efficiently to erythrocytes with high CR1 number (Fig. 3).

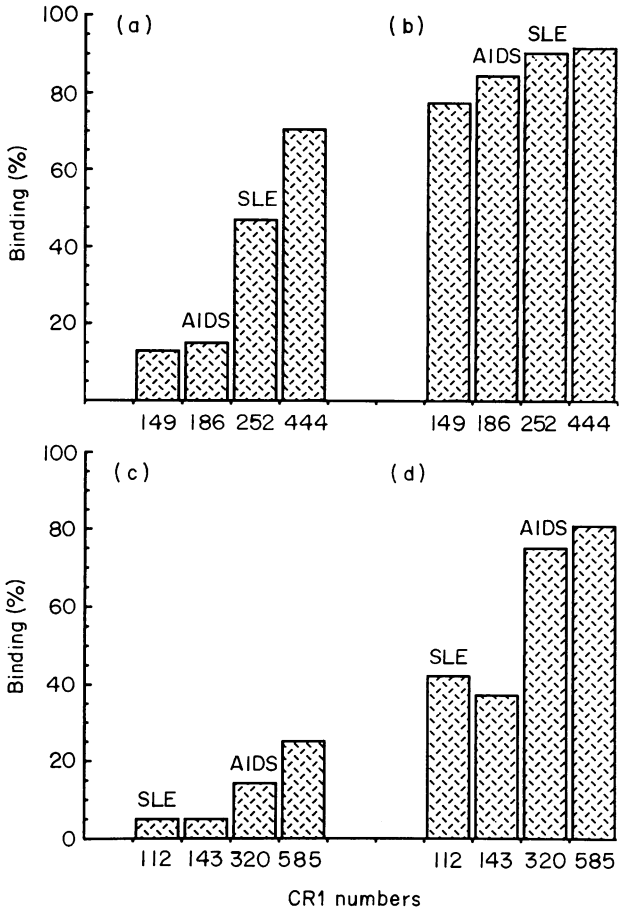

Fig. 1. Binding of immune complexes (%) after an incubation for 2 h at 4°C (a, c) and 37°C (b, d) onto erythrocytes bearing different CR1 numbers. The experiments were performed in excess of CR1 (maximal binding), but at equal CR1 numbers. (a, b) High MW immune complexes (c, d) low MW immune complexes. The binding was almost identical at 37°C for large immune complexes, however, differences became apparent for small immune complexes and when the incubations were performed at 4°C.

Finally we assessed the effect of adding a monoclonal anti-CR1 antibody(3D9) known to block immune complex binding to erythrocytes. Addition of such a MoAb should reduce homogeneously the number of CR1 sites/CR1 cluster. Thus it should have less effect on large clusters with abundant C3b binding sites than on small clusters: 50% blockade of a cluster of four CR1 might render the cluster incapable of binding an immune complex, whereas an identical blockade of a cluster with eight CR1 would not have this effect. More MoAb was needed to abolish binding to cells with high CR1 number than to cells with low CR1 (Fig. 4). This observation might be taken as the functional equivalent of the reduced clustering on cells bearing few CR1.

The cells used in the previous experients expressed the AA phenotype. Every one of the experiments shown was confirmed using at least three different pairs of erythrocytes from normal individuals with high versus low CR1. Erythrocytes from five SLE and three AIDS subjects with reduced CR1 were compared with normal erythrocytes and all showed low efficiency for immune complex binding (kinetics at 37°C, and 2 h at 4°C)

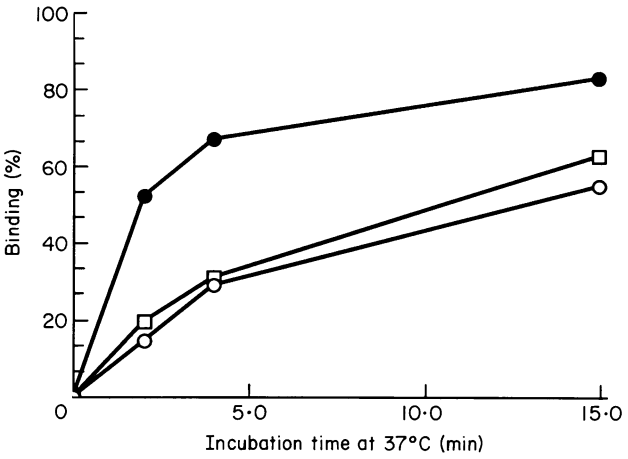

Fig. 2. Kinetics of immune complex binding (%) to erythrocytes bearing different CR1 numbers (●, 456; □, 186 (AIDS); and ○, 146). This experiment was performed at 37°C, in excess of CR1 and at equal CR1 concentration. Immune adherence was fastest on cells bearing high CR1 number.

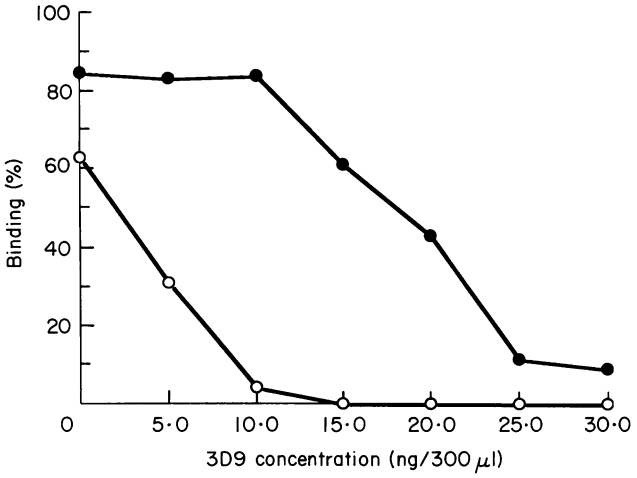

Fig. 4. Inhibition of immune adherence of immune complexes by MoAb 3D9. The MoAb was incubated first with the cells for 30 min before adding the immune complexes. Blocking identical numbers of CR1 interfered more efficiently with the binding of immune complexes to cells bearing low CR1 number.

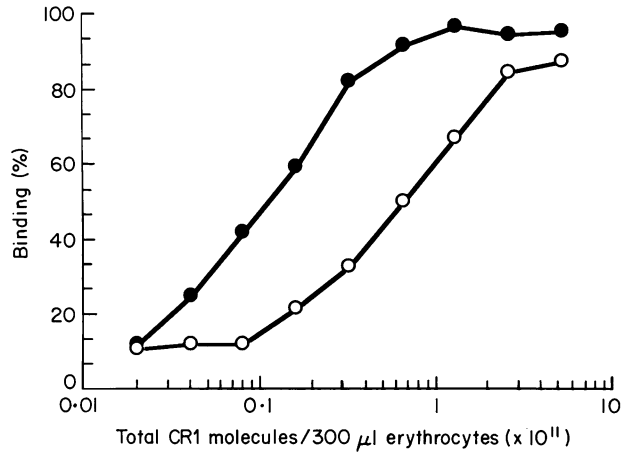

Fig. 3. Binding (%) of immune complexes to erythrocytes bearing different CR1 numbers (●, 430; and ○, 161) at 37°C after 30 min. The difference in binding efficiency was apparent over a wide range of final CR1 concentration (from excess immune complexes to excess CR1).

Table 1. Binding of immune complexes to erythrocytes with different CR1 alleles

		Binding (%)*					
		2 h, 4°C		2 min, 37°C		2 h, 37°C	
Alleles	CR1/erythrocytes	1	2	1	2	1	2
AA	277	48	49	35	34	82	81
DD	231	55	62	41	36	80	78
AA	540	53	57	44	43	80	80
BB	569	71	70	48	49	79	81

Experiment performed in CR1 excess, at equal CR1 concentration.
* Each value was measured in duplicate (variation of binding ≤2%);
Each experiment was performed using two different batches of immune complexes: (1 and 2).

which was in the same range as normal erythrocytes with similar CR1 numbers (see Figs 1 and 2).

In order to analyse further the role of alleles in the efficiency of immune complex binding, we selected donors bearing different CR1 alleles (homozygous AA, BB and DD), but with almost identical numbers of CR1 (Table 1). Both DD and BB were more efficient at binding immune complexes than the corresponding AA control erythrocytes at 4°C. The differences after 2 min at 37°C were less apparent, and, as expected, disappeared after incubation for 2 h at 37°C.

To study further the effect of CR1 alleles we analysed the binding of the same immune complexes to erythrocytes by immunofluorescence. A series of experiments was performed to define this new technique. First, the binding was assessed after incubation for 1 h at 37°C in excess of immune complexes, i.e. adding enough immune complexes so that only 20% (±5%) of the offered immune complexes bound to the erythrocytes. Under such saturating conditions, erythrocytes carried 0–15 immune complexes, assuming that one IF dot corresponded to one immune complex (Fig. 5a). There was always an extreme heterogeneity in the immune complex binding to the erythrocytes which corresponds probably to the known heterogeneity of CR1 clusters on erythrocytes (Paccaud *et al.*, 1988). A significant fraction of erythrocytes did not bind immune complexes, and this fraction could not be diminished by either reducing the concentration of erythrocytes four-fold with only 6% of the immune complexes being bound, or by exposing these cells twice to excess immune complexes. To see whether immune complexes rearrange after their binding to erythrocytes, cells were incubated either 10 min or 60 min with immune complexes;

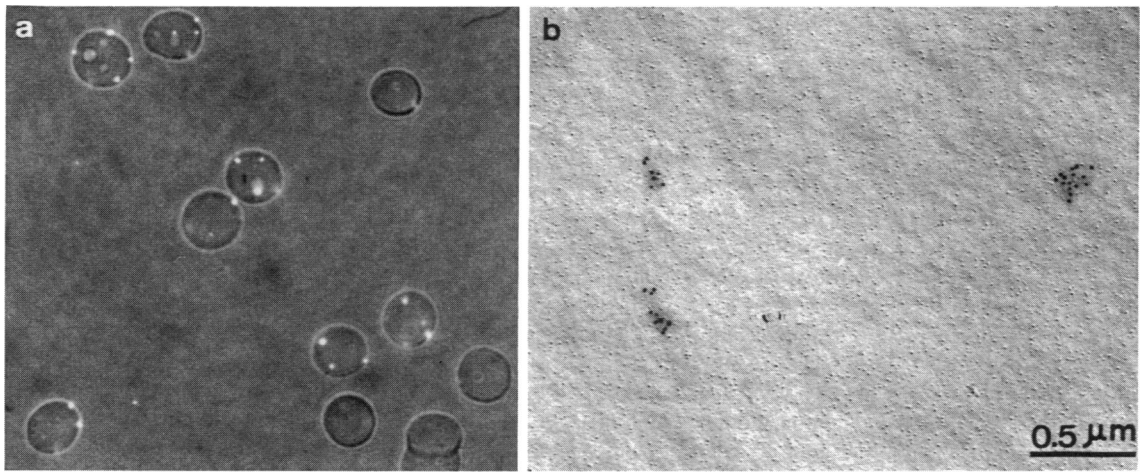

Fig. 5. (a) Immunofluorescence with phase contrast microscopy of erythrocytes bearing immune complexes. Fluorescent dots corresponded to immune complexes; (b) immuno-gold electron microscopy showing three clusters of gold particles corresponding to three immune complexes.

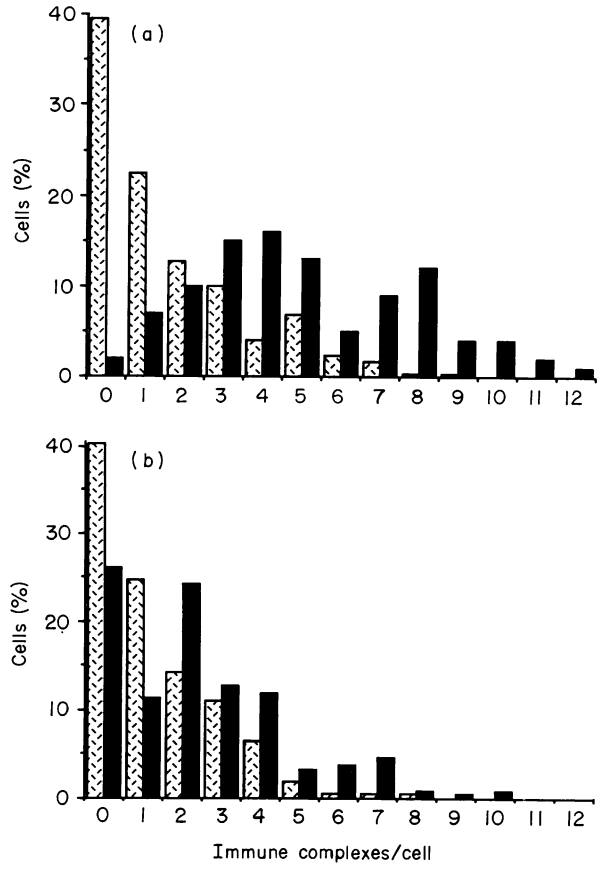

Fig. 6. Distribution of immune complexes on erythrocytes bearing (a) different numbers of CR1 of AA types (▨, low; ■, high); (b) DD (■) *versus* (■) alleles with the same number of CR1/erythrocytes (278 and 285 CR1/erythrocytes, respectively). (a) The erythrocytes with 954 (high) CR1 bound a mean of 5·00 immune complexes/cell, whereas those with 240 (low) CR1 bound only 1·59 immune complexes/cell (significant difference in the number of immune complexes per cell by the Mann–Whitney *U*-test: $P=0{\cdot}0001$), (b) the erythrocytes with the DD allele bound 2·36 immune complexes/cell, those with the AA allele bound 1·03 immune complexes/cell (Mann–Whitney *U*-test: $P=0{\cdot}0001$).

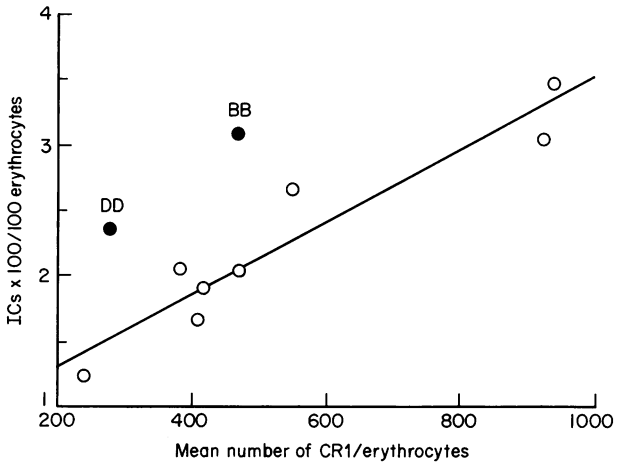

Fig. 7. Number of immune complexes (ICs) fixed on 100 erythrocytes using cells with AA (open circles), BB or DD alleles and different CR1 numbers (experiments performed for all cells on the same day). The increase in efficiency of immune complexes was linear for AA alleles ($r^2=0{\cdot}905$, $P<0{\cdot}0005$). The cells bearing DD or BB aleles were more efficient.

under both conditions $19\pm2\%$ of the immune complexes were bound, and the distribution of immune complexes on erythrocytes by IF was not modified (mean immune complex number/cell $=3{\cdot}6\pm0{\cdot}2$ and $3{\cdot}5\pm0{\cdot}2$, respectively). Erythrocytes were then incubated for 2 min with immune complexes, washed and further incubated at 37°C for 1 h. Less than 5% of the immune complexes were released, and again no rearrangements could be demonstrated. Cells were prefixed with either formaldehyde or carboidimide. Both fixation procedures reduces the binding capacity of immune complexes by 10–15%, and the CR1 sites defined by E11 or 3D9 MoAbs also be approximately 10%. However, the distribution of immune complexes on such fixed cells was similar to unfixed cells, although again slightly reduced. By electron microscopy, each complex was revealed by a cluster of gold particles (Fig. 5b); the heterogeneity in numbers of gold particles corresponded probably to differences in the

accessibility or number of C3 antigenic sites available for the specific antibody used. This might also explain the difference in the intensity of IF dots. Only a few such clusters of gold particles could be observed on one erythrocyte. Control experiments indicated that these clusters were specific for complement-opsonized immune complexes.

When analysed by IF, the binding of immune complexes to erythrocytes with different CR1 number showed on erythrocytes with high CR1 number, better mean binding to erythrocytes, a higher maximal number of immune complexes/cell and less erythrocytes devoid of immune complexes (Fig. 6a). Such differences were observed in three different pairs studied, although there was some daily variation in the results obtained. However, for experiments performed under identical conditions (same CR1 concentration, and same immune complex binding by RIA), the mean number of immune complexes/erythrocytes did not increase to the expected level, i.e. cells bearing three-fold more CR1 did not bind more than two-fold more immune complexes by IF (Fig. 7). This suggests that defining one IF dot as one immune complex might be an over-simplification.

The binding of immune complexes to cells bearing the DD or the BB alleles was clearly above what would have been expected for their CR1 number (significant differences by the Mann–Whitney *U*-test: both $P=0{\cdot}0001$) (Fig. 6b). The respective role of CR1 number *versus* alleles is illustrated in Fig. 7.

DISCUSSION

The results obtained demonstrate that both CR1 numbers and alleles affect the immune complex binding efficiency of CR1 on erythrocytes.

That clustering of C3b binding sites of CR1 is a prerequisite for efficient complex binding has been suggested for several reasons. Firstly, the binding affinity of immune complexes for erythrocyte CR1 is several orders of magnitude higher than that of monomeric C3b (Arnaout *et al.*, 1981; Schifferli *et al.*, 1988). Secondly, the binding of immune complexes requires optimal fixation of multiple C3b onto large immune complexes (Lennek *et al.*, 1981; Edberg *et al.*, 1988). Thirdly, CR1 form clusters on human erythrocytes (Paccaud *et al.*, 1988). Finally, Cosio, Yiao-Peng & Herbert (1990) showed that fluorescent beads coated with MoAb against CR1 bound to a fraction of the erythrocytes (1–4 beads/erythrocyte). This binding of fluorescent beads reduced CR1 sites on the erythrocytes by only 30% but abolished the binding of immune complexes, suggesting again that only some CR1 are responsible for immune complex binding, probably those forming large clusters. The first direct evidence that clustering was essential for immune complex binding came from the observation of poor binding efficiency of CR1 on polymorphonuclear leucocytes (PMN) compared with erythrocytes (Paccaud, Carpentier & Schifferli, 1990). Although resting and activated PMN expressed on their cell membrane several-fold more CR1 than erythrocytes, binding efficiency, as determined from the immune complex binding/CR1 ratio, was significantly lower for PMN. Morphological analysis showed much less clustering of CR1 on PMN than on erythrocytes. Since erythrocytes with low CR1 show also reduced clustering of CR1 (Paccaud *et al.*, 1988), we compared the binding of immune complexes to erythrocytes with high and low CR1, correcting for the CR1 number so as to provide identical final CR1 concentrations. The experiments performed were all concordant: a low number of CR1 meant also a reduced immune complex binding efficiency, which is in accordance with reduced clustering. Of interest was the observation that cells from patients with acquired low CR1 (SLE, AIDS), showed also reduced binding efficiency which corresponded to their CR1 number. Thus, there was no demonstrable loss of specific immune complex binding sites.

A second way of increasing C3b binding sites is to have more such sites per CR1. The data from Bartow *et al.* (1989) would suggest that the structural alleles of CR1 have between two and five C3b-binding sites. Wong, Wilson & Fearon (1982) showed that C3b dimers have almost identical affinity for the different structural alleles of CR1, although fine analysis of the data indicates that larger alleles were slightly more efficient in binding C3b dimers. The differences demonstrated here in the table indicate similar trends. However, the observations made using IF techniques in the presence of excess immune complexes provided clear evidence that CR1 alleles determine also the binding efficiency of CR1: the larger the allele, the more efficient the binding was. Whether this was due to the binding of several C3b to one CR1 molecule, or to an easier cross-linking of two CR1 molecules of larger size by one immune complex is presently unknown.

Determination of CR1 with MoAbs might lead to overestimation of CR1 molecules/erythrocytes because of the repetitive structure of CR1. This artefact would not influence our results obtained with erythrocytes bearing identical alleles (AA); however, it might lead to a disproportionally higher estimation of CR1 for cells bearing the larger alleles (BB and DD). Thus the real differences between binding efficiences of DD and BB *versus* AA alleles might be even higher.

Analysis of immune complex binding by IF provided a new approach to define possible rearrangements of immune complexes which might take place on erythrocytes. No such rearrangements could be demonstrated using cells fixed with formaldehyde or carboidimide, or increasing immune complex concentration or incubation times. This does not exclude some minor re-organization since, enumeration of immune complexes by IF did not relate directly to radioactivity measurements. However, major redistribution of immune complexes was excluded, and the results obtained might be explained by some complex aggregation via Ag/Ab bonds or Fc–Fc interactions between fixed and soluble immune complexes. Alternatively, some large CR1 clusters might fix two or more immune complexes, or a systematic error might be introduced when cells bearing many immune complexes are analysed, and the counting of fluorescent dots becoming more difficult.

NOTE ADDED IN PROOF

The erythrocytes described as DD were shown to contain only the D allele at the time of our study. However, at a later stage a faint A allele also became apparent, indicating heterozygousy (AD).

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